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<http://dx.doi.org/10.1111/mec.14456>

<b>Title</b>	Probiotic consortia are not uniformly effective against different amphibian chytrid pathogen isolates
<b>Authors</b>	Antwis, RE and Harrison, XA
<b>Type</b>	Article
<b>URL</b>	This version is available at: <a href="http://usir.salford.ac.uk/44467/">http://usir.salford.ac.uk/44467/</a>
<b>Published Date</b>	2017

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## ORIGINAL ARTICLE

# Probiotic consortia are not uniformly effective against different amphibian chytrid pathogen isolates

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**Funding information**

University of Salford Pump Priming Fund; Research Fellowship, North-West University, South Africa; Research Fellowship, Institute of Zoology, Zoological Society of London

**Abstract**

Symbiotic bacterial communities can protect their hosts from infection by pathogens. Treatment of wild individuals with protective bacteria (probiotics) isolated from hosts can combat the spread of emerging infectious diseases. However, it is unclear whether candidate probiotic bacteria can offer consistent protection across multiple isolates of globally distributed pathogens. Here, we use the lethal amphibian fungal pathogen *Batrachochytrium dendrobatidis* to investigate whether probiotic richness (number of bacteria) or genetic distance among consortia members influences broad-scale in vitro inhibitory capabilities of probiotics across multiple isolates of the pathogen. We show that inhibition of multiple pathogen isolates by individual bacteria is rare, with no systematic pattern among bacterial genera in ability to inhibit multiple *B. dendrobatidis* isolates. Bacterial consortia can offer stronger protection against *B. dendrobatidis* compared to single strains, and this tended to be more pronounced for consortia containing multiple genera compared with those consisting of bacteria from a single genus (i.e., with lower genetic distance), but critically, this effect was not uniform across all *B. dendrobatidis* isolates. These novel insights have important implications for the effective design of bacterial probiotics to mitigate emerging infectious diseases.

**KEYWORDS**amphibians, bacteria, *Batrachochytrium dendrobatidis*, emerging infectious disease, phylogeny, probiotics

## 1 | INTRODUCTION

The last 50 years has seen the emergence of several virulent wildlife pathogens with broad host ranges (Tompkins, Carver, Jones, Krkošek, & Skerratt, 2015). These emerging infectious diseases have decimated wildlife populations globally and are major contributors to the current global loss of biodiversity (e.g., McCallum, 2012; Skerratt et al., 2007). Broad-scale treatments and/or prophylaxis for such pathogens are often lacking for wild animals (Garner et al., 2016;

Sleeman, 2013). Developing such treatments is often complicated by broad variation in genetic and phenotypic traits such as virulence exhibited by these pathogens (e.g., Farrer et al., 2011; de Jong & Hien, 2006; Schock, Bollinger, & Collins, 2009). Successful mitigation of infectious diseases in the wild demands that preventative or curative therapies demonstrate broad activity over as many genetic variants of the pathogen as possible, and developing mitigation strategies that satisfy this criterion remains a major outstanding research goal.

*Batrachochytrium dendrobatidis* is a highly infectious fungal pathogen responsible for the global decline in amphibians and a major

Antwis and Harrison contributed equally to this study.

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driver of the current “amphibian extinction crisis” (reviewed in Garner et al., 2016). This pathogen comprises multiple deeply diverged lineages and is capable of rapid evolution through extensive genomic recombination (Farrer et al., 2011, 2013). Endemic hypovirulent lineages of *B. dendrobatidis* have been identified including BdCAPE (South Africa), BdCH (Switzerland), BdBrazil (Brazil) and a lineage from Japan (Farrer et al., 2011; Goka et al., 2009; Rodriguez, Becker, Pupin, Haddad, & Zamudio, 2014; Rosenblum et al., 2013; Schloegel et al., 2012), although such endemic lineages may be implicated in population declines in novel regions (e.g., BdCAPE in Mallorcan midwife toads, *Alytes muletensis*; Doddington et al., 2013). However, it is the globally distributed hypervirulent global panzootic lineage (BdGPL) that is associated with phenomenal mass mortalities and rapid population declines of amphibians around the world (Farrer et al., 2011; Fisher et al., 2009; Olson et al., 2013). Isolates within this lineage exhibit enormous and unpredictable variation in virulence, even within a single host species exposed under laboratory conditions (Farrer et al., 2011, 2013).

There is currently no cure for chytridiomycosis in the wild (reviewed in Garner et al., 2016). Given that amphibian communities may be host to multiple *B. dendrobatidis* variants (Morgan et al., 2007; Rodriguez et al., 2014) and that global movement of humans and wildlife continues to transport the pathogen (Garner et al., 2016), any prophylactic or curative treatment needs to be effective against multiple *B. dendrobatidis* variants. Bacterial probiotics represent a promising tool to combat emerging infectious diseases in the wild, including *B. dendrobatidis* (Bletz et al., 2013; Hoyt et al., 2015; Rebollar et al., 2016). Laboratory and field studies have shown host-associated bacterial communities protect amphibians from *B. dendrobatidis* infection and that it is possible to artificially augment the microbiota with probiotic bacteria to improve survivorship in response to the pathogen (Becker et al., 2015; Bletz et al., 2013; Harris, Lauer, Simon, Banning, & Alford, 2009; Jani & Briggs, 2014; Kueneman et al., 2016; Mulet, Myers, Domangue, Herrick, & Harris, 2012; Walke et al., 2015). However, inhibitory capabilities of individual bacteria are not uniform across the variation presented by *B. dendrobatidis* (Antwis, Preziosi, Harrison, & Garner, 2015; Bletz, Myers, et al., 2017; Mulet-Wolz et al., 2017). In addition, previous work has found either no (Becker et al., 2015) or weak evidence (Bletz, Myers, et al., 2017) of a phylogenetic signal in the ability of bacterial genera to inhibit a singular *B. dendrobatidis* isolate. However, a major gap in our understanding concerns whether some bacterial genera are more likely to show broad-spectrum inhibition across a range of *B. dendrobatidis* isolates, allowing a more focused search for effective amphibian probiotics.

Furthermore, the importance of a complex and diverse microbiota for resilience to infection has been repeatedly demonstrated across a range of host taxa (e.g., Dillon, Vennard, Buckling, & Charnley, 2005; Eisenhauer, Schulz, Scheu, Jousset, & Pfender, 2013; van Elsas et al., 2012; Matos, Kerkhof, & Garland, 2005). An alternative strategy for probiotic development involves a “bacterial consortium” approach, whereby multiple inhibitory bacterial strains are applied simultaneously. Multispecies consortia can increase inhibition of

*B. dendrobatidis* growth through increased competition and the production of emergent metabolites (Loudon et al., 2014; Piovra-Scott et al., 2017) and may offer greater inhibitory capabilities across a wider range of *B. dendrobatidis* isolates. However, the generality of this pattern across multiple pathogen variants remains untested. Addressing this shortfall in our understanding is critical for developing effective tools for the mitigation of emerging infectious diseases in the wild.

Here, we extend previous work to quantify the ability of individual bacteria and cocultured bacterial consortia to demonstrate broad-scale inhibition across a panel of *B. dendrobatidis* isolates. First, we test 54 bacterial strains from 10 genera for inhibition against a suite of 10 different BdGPL isolates to quantify: (i) variation among bacterial genera in ability to demonstrate broad-spectrum BdGPL inhibition; and (ii) variation among BdGPL isolates in susceptibility to inhibition. Second, we quantify the relative efficacy of using single bacterial strains or bacterial consortia to modify *B. dendrobatidis* growth rates in vitro. Specifically, we investigate (iii) whether consortia yield stronger inhibition than single bacterium across three *B. dendrobatidis* isolates from two lineages (BdGPL and BdCAPE); and (iv) whether genus-level diversity of a bacterial consortium affects inhibitory capabilities.

## 2 | METHODS

### 2.1 | Taxonomic classification

In vitro challenges were conducted for 54 bacteria isolated from wild *Agalychnis* spp. frogs in Belize (Antwis et al., 2014, 2015; Woodhams et al., 2015) to screen for inhibitory capabilities against 10 BdGPL isolates (Table 1, Figure 1). *Batrachochytrium dendrobatidis* is present in the Maya Mountains from where these bacteria were collected, although declines in *Agalychnis* hosts were not seen in this area (Kaiser & Pollinger, 2012; R. E. Antwis, personal observation). Bacterial strains belonged to 10 genera with 3–11 bacteria per genus (Table S1). Bacteria were identified using colony PCR to amplify the 16S rRNA gene (with primers 27F and 1492R) and sequenced at the University of Manchester (Antwis et al., 2014). The forward and reverse sequences were aligned for each bacterium and blasted against the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To calculate genetic distance among sequences, we aligned the sequences against the SILVA reference database (Quast et al., 2013). We used the *seqinr* package (Charif & Lobry, 2007) to import the aligned sequences and calculate the pairwise genetic distances among bacterial strains.

Inhibition challenges were conducted using an in vitro absorbance-based growth inhibition assay adapted from Bell, Alford, Garland, Padilla, and Thomas (2013), Woodhams et al. (2014) and Becker et al. (2015). Bacteria were grown by adding 50 µl of frozen stock bacteria (stored in 30% glycerol, 70% tryptone solution at –80°C) to 15 ml of 1% tryptone and incubating at 18°C for 36 hr until turbid (three cultures per bacterial strain). Although cell density has been shown to influence metabolite production in culture (Yasumiba, Bell, & Alford, 2016), we decided not to count and adjust

**TABLE 1** *Batrachochytrium dendrobatidis* isolates used in the study

Isolate	Lineage	Geographic	Host species	Collector	Year	Phylogeny screening	Consortium challenges
MG04	GPL	Silver Mine, Western Cape, South Africa	<i>Amietia fuscigula</i>	Trenton Garner	2010	X	
CORN2.2	GPL	Penhale Farm, Cornwall, UK	<i>Ichthyosaurus alpestris</i>	Trenton Garner	2012	X	
CORN2.3	GPL	Penhale Farm, Cornwall, UK	<i>Ichthyosaurus alpestris</i>	Trenton Garner	2012	X	
CORN3.1	GPL	Penhale Farm, Cornwall, UK	<i>Ichthyosaurus alpestris</i>	Trenton Garner	2012	X	
CORN3.2	GPL	Penhale Farm, Cornwall, UK	<i>Ichthyosaurus alpestris</i>	Trenton Garner	2012	X	
AUL1.2	GPL	Lac d'Aule, France	<i>Alytes obstetricans</i>	Matthew Fisher	2010	X	
AUL2	GPL	Lac d'Aule, France	<i>Alytes obstetricans</i>	Matthew Fisher	2010	X	
IA2011	GPL	Ibon Acherito, Spain	<i>Alytes obstetricans</i>	Matthew Fisher	2011	X	
MODS 28.1	GPL	Mont Oliu, Sardinia	<i>Discoglossus sardus</i>	Trenton Garner	2010	X	
JEL423	GPL	Guabal, Panama	<i>Agalychnis lemur</i>	Joyce Longcore	2004	X	X
SFBC019	GPL	Sellafield, Cumbria, UK	<i>Epidalea calamita</i>	Peter Minting	2010		X
TF5a1	CAPE	Torrent des Ferrerets, Mallorca	<i>Alytes muletensis</i>	Matthew Fisher	2007		X

cell density prior to inhibition trials as subsequent addition of media may alter the metabolite profiles already produced by cultures. In addition, cultures were not grown in the presence of *B. dendrobatidis* as multiple *B. dendrobatidis* isolates were tested in this study.

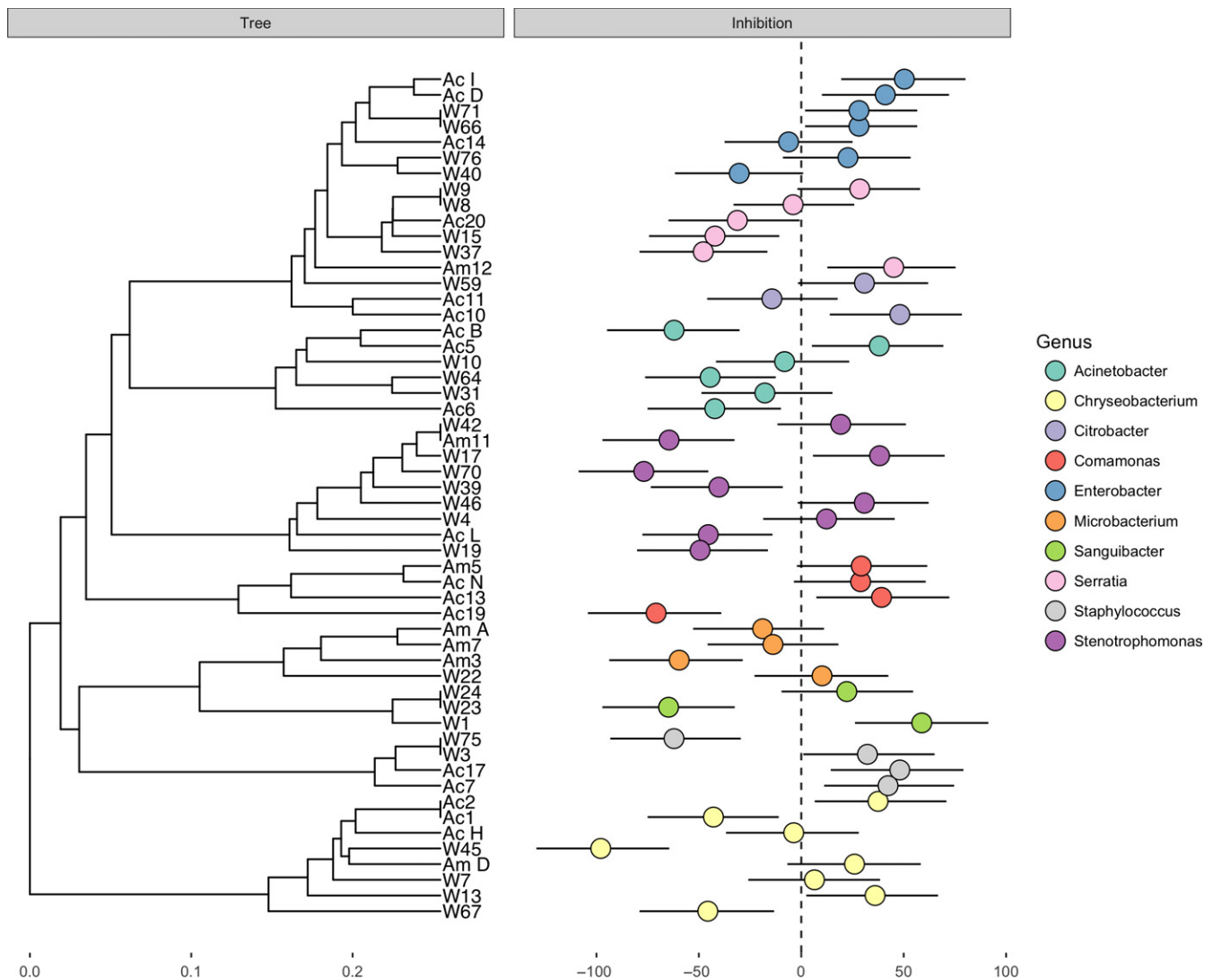
Turbid cultures were filtered through a 0.22- $\mu$ m sterile filter (Millipore, Ireland) to remove live cells, leaving only bacterial products (including metabolites) in the filtrate. These were then combined across the three cultures for a given bacterial strain and kept on ice until *B. dendrobatidis* challenges were conducted. *BdGPL* (Table 1) isolates were grown in 1% tryptone broth until maximum zoospore production was observed (~3–4 days;  $\sim 1 \times 10^6$  zoospores  $\text{ml}^{-1}$ ). As with bacteria, three flasks per *B. dendrobatidis* isolate were grown and then combined prior to challenges to minimize flask effect. Zoospores were separated from sporangia by filtering through 20- $\mu$ m sterile filters (Millipore, Ireland). To conduct the absorbance-based growth inhibition assays, 50  $\mu$ l of bacterial filtrate and 50  $\mu$ l of *B. dendrobatidis* suspension were pipetted into 96-well plates. Each *B. dendrobatidis*–bacteria combination was run in triplicate. Positive controls were included using 50  $\mu$ l 1% tryptone instead of bacterial filtrate. Negative controls were included using 50  $\mu$ l of sterile tryptone and 50  $\mu$ l of heat-treated *B. dendrobatidis* for each isolate. Plate readings were taken using a 492-nm filter on initial construction of the challenge assays and every 24 hr for four subsequent days.

For each measurement, data were transformed using the equation  $\text{Ln}(\text{OD}/(1-\text{OD}))$  and a regression analysis was used to gain the

slope values for each sample over time. Slopes of triplicate replicates for each *B. dendrobatidis*–bacteria combination were averaged, and the slope of the negative controls subtracted. Total *B. dendrobatidis* inhibition was calculated using the formula: Inhibition (%) =  $[1 - (\text{slope of sample}/\text{slope of control})] \times 100$  to give an “inhibition score.” A positive inhibition score represents inhibition of *B. dendrobatidis* growth and a negative score indicates enhanced growth of *B. dendrobatidis*. It should be noted that we did not use a nutrient-depleted control in our experiments (Bell et al., 2013), which means *B. dendrobatidis* inhibition relative to the controls may be slightly underestimated.

## 2.2 | Bacterial consortium challenges

Three bacterial strains were then selected from each of five genera (*Chryseobacterium*, *Comamonas*, *Enterobacter*, *Staphylococcus* and *Stenotrophomonas*) based on their inhibition profiles; poor to medium inhibitors were selected to determine whether combining these bacteria would improve their inhibitory capabilities (mean percentage inhibition score of approximately 0 to +50; Figure 1). Bacteria were grown individually until turbid and added to fresh tryptone either individually (strains A, B and C of each genus separately) or as a triple (strains A, B and C of each genus together to form five single-genus mixes or a combination of strains across genera to form multi-genus consortia, with a total of 20 multigenus combinations tested). For both individual and triple bacterial combinations, a total of 3 ml



**FIGURE 1** Inhibition scores of 54 bacterial strains from 10 genera when tested against 10 *BdGPL* isolates. A positive value represents inhibition of *Batrachochytrium dendrobatidis* growth and a negative value indicates enhanced growth of *B. dendrobatidis*. Estimates are derived from a Bayesian mixed-effects model with bacterial strain nested within genus, and *BdGPL* isolate fitted as random effects. Points are conditional modes of the individual bacterial strain random effects, marginalized with respect to *BdGPL* isolate. Error bars are 95% credible intervals. Bacterial strains from the same genus are denoted by the same colour

of bacteria were added to 12 ml of fresh 1% tryptone broth and left to grow together for 12 hr. The volume of each bacterium added depended on whether the consortium contained one or three bacteria, and the volume was split evenly between the numbers of bacteria added to each group. Following this, bacteria–*B. dendrobatidis* challenges were conducted using the same methods as described above against three *B. dendrobatidis* isolates (Table 1). Average inhibition percentages for each consortium–*B. dendrobatidis* combination were calculated as described above.

## 2.3 | Statistical analysis

All statistical analyses were conducted in the software R v.3.3.2 (R Core Team 2016).

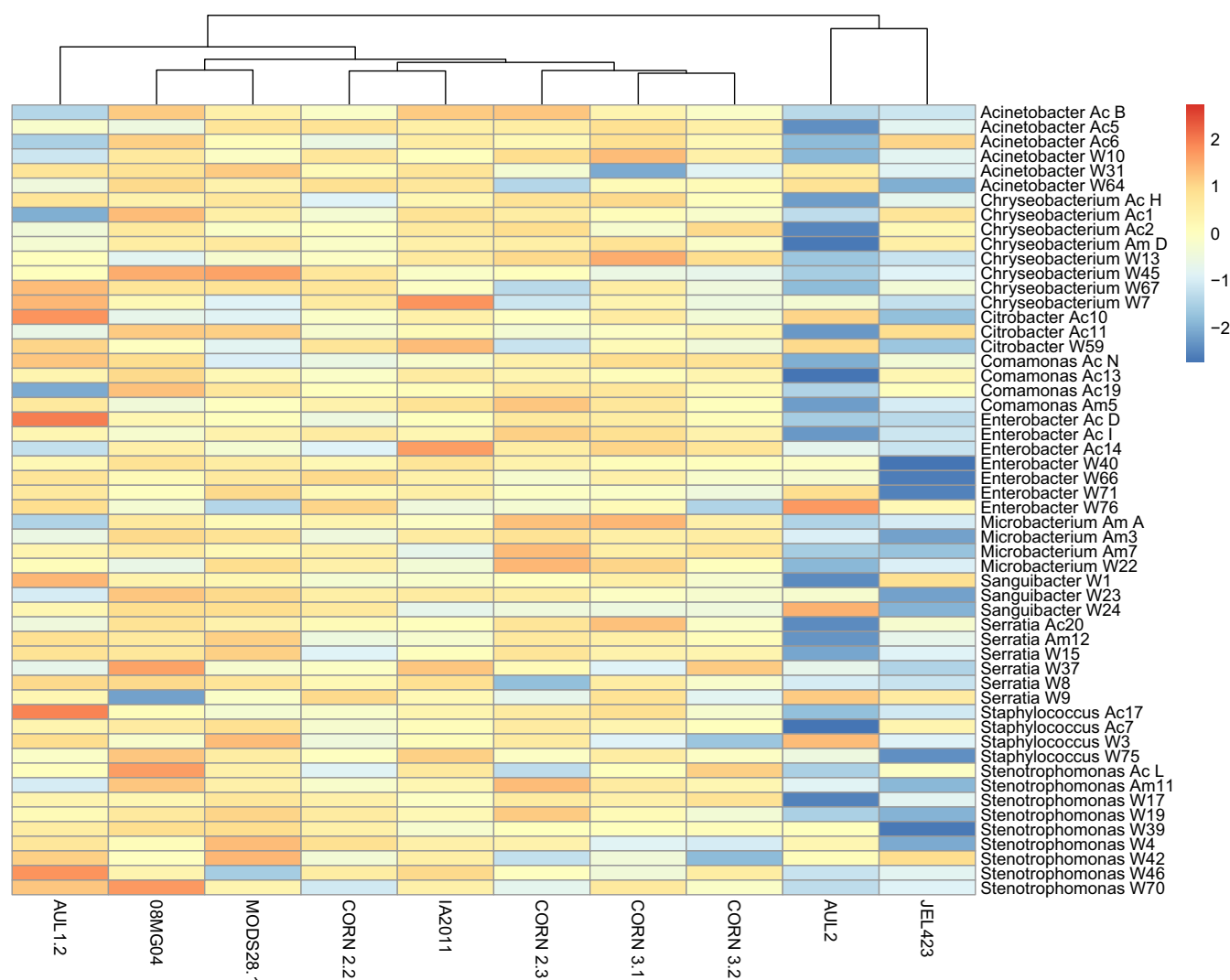
### 2.3.1 | Taxonomic group data

To quantify differences among genera in proportion of *BdGPL* isolates inhibited (i.e., for those where inhibition score >0), we fitted a binomial GLM with the proportion of the 10 *BdGPL* isolates each bacterial strain inhibited as the response, and genus as a fixed effect. We used the quasibinomial error structure as the model was overdispersed (dispersion 6.4) and tested the model containing a genus term with the reduced intercept-only model using a likelihood ratio test.

To visualize the distribution of inhibition across bacterial strains and *B. dendrobatidis* isolates, we constructed a heatmap using the *pheatmap* package in R (Kolde, 2015). To quantify differences among genera in the degree of inhibition (size of inhibition score), we fitted

a hierarchical model in the R package *MCMCglmm* (Hadfield, 2010) with the individual inhibition scores of each bacterial strain ( $n = 54$ ) for each *BdGPL* isolate ( $n = 10$ ; total  $n = 540$ ) as a Gaussian response. We fitted both *BdGPL* isolate and bacterial strain ID nested within bacterial genus as random effects. We also controlled for genetic distance among bacterial strains by passing the bacterial 16S gene tree to the model as a phylogenetic random effect. We use uninformative, parameter-expanded priors for the random effects as detailed in Hadfield (2010). We ran models for a total of 100,000 iterations following a burn-in of 10,000 iterations and using a thinning interval of 50. Inspection of model residuals from the frequentist analogue of this model fitted in *lme4* (Bates, Maechler, Bolker, & Walker, 2015) revealed normally distributed residuals and no evidence of heteroscedasticity. Rerunning models with stronger priors has no effect on model results. Gelman–Rubin diagnostic of Markov chains indicated adequate convergence, with all potential

scale reduction factors  $<1.01$ . We used Bayesian models here, rather than a frequentist analogue, due to the ease of summarizing uncertainty in point estimates of random effect conditional means using 95% credible intervals of Markov chain values. To calculate % variance in inhibition explained by *BdGPL* isolate, bacterial genus and bacterial strain respectively, we extracted the variance components from the variance–covariance matrix of the model above. We expressed the variance of a component  $V$  as a percentage of the total variance calculated as  $(V_{\text{BdGPL}} + V_{\text{genus}} + V_{\text{strain}} + V_{\text{residual}})$ . We calculated both mean and 95% credible intervals using the posterior samples from the model. To construct Figures 1 and 2, we extracted the marginal means and 95% credible intervals for each bacterial strain and *BdGPL* isolate, respectively. That is, the bacterial strain modes are marginalized with respect to *BdGPL*, and vice versa, to quantify whether the average scores for each *BdGPL* isolate or bacterial strain are significantly different from zero.



**FIGURE 2** Heatmap of inhibition across all 54 bacterial strains and all 10 *Batrachochytrium dendrobatidis* isolates. Bacterial strains have been clustered according to phylogeny, and *B. dendrobatidis* isolates have been clustered according to their similarity in inhibition profiles (dendrograms in left and top margins, respectively). Inhibition scores have been z-transformed across *B. dendrobatidis* isolates (rows) for each particular bacterial strain. Bacterial row names include both genus and strain ID. Blue indicates low inhibition through to red, which indicates high inhibition



### 2.3.2 | Correlation between genetic distance and inhibition

For each pair of bacterial strains, we calculated the correlation between the inhibition scores for the ten *B. dendrobatidis* isolates. If more closely related bacterial strains are more likely to have similar inhibition profiles, there should be a negative correlation overall between genetic distance and similarity of inhibition. To test this, we performed a Mantel test using the genetic distance and inhibition score similarity matrices in the R package “vegan” (Oksanen et al., 2015).

### 2.3.3 | Consortium data

To calculate the relative mean inhibition of single-genus vs. multi-genus consortia, we fitted a mixed model in *MCMCglmm* with inhibition as a Gaussian response, consortium type as a two-level factor and a random effect of *B. dendrobatidis* isolate using uninformative priors. To calculate whether consortia exhibited stronger inhibition than the mean of their individual strains, we constructed a binary variable with an outcome of 1 if a consortium's inhibition was greater than the single strain mean, and 0 if equal to or lower. We fitted this as a response in a binary GLMM with consortium type as a fixed effect, *B. dendrobatidis* as a random effect and using uninformative priors. Neither model exhibited signs of autocorrelation and Geweke statistics for both models indicated convergence (Geweke, 1992). We calculated mean genetic distance among members of consortia using the genetic distance measures outlined above. We fitted a Bayesian GLM where the percentage inhibition of a consortium was a function of the interaction between the genetic distance among consortia members and the *B. dendrobatidis* isolate identity. Genetic distance was standardized prior to model fitting to remove the correlation between main effects and interactions.

### 2.3.4 | Consortium randomizations

We used a randomization approach to probe the relative effectiveness of single bacteria, single-genus consortia and multigenus consortia (hereafter “probiotic types”) for modifying the growth rates of *B. dendrobatidis*. These randomizations used the “Taxonomic Group” and “Consortium” inhibition data from above to explore three different scenarios relevant to the application of probiotics to *B. dendrobatidis*. For each iteration of a randomization, we randomly selected a *B. dendrobatidis* isolate and then extracted the inhibition scores of a randomly chosen single bacterial strain, single-genus consortium and multigenus consortium. After 1,000 iterations, we calculated (i) the proportion of times a multigenus consortium yielded higher inhibition than a single-genus consortium; (ii) the proportion of times a multigenus consortium yielded higher inhibition than a single bacterial strain; and (iii) the probability that a multigenus, single-genus or single bacterial strain would yield at least 50% inhibition, which we classed as strong inhibition. This approach is more powerful than simply calculating differences in group means of each probiotic type, as group means can be skewed by large individual values, and therefore be

misleading with respect to the efficacy of a particular strategy if the mean of that group is not reflective of the true variance in the data. However, we report group means alongside these statistics where appropriate for comparison. We derived 95% confidence intervals for each test statistic by performing 10,000 bootstrap samples with replacement from the test distributions. The three scenarios we tested were as follows:

**Scenario 1:** Averaged over all *B. dendrobatidis* isolates: For each iteration, we randomly selected a *B. dendrobatidis* isolate and then randomly selected both a single-genus and a multigenus consortium. A single bacterial strain score was then selected randomly from one of the members of the multigenus consortium.

**Scenario 2:** *B. dendrobatidis*-specific scores: To investigate the potential for the effectiveness of consortia to differ depending on *B. dendrobatidis* isolate, we repeated the randomization as in Scenario 1 but performed 1,000 iterations for each *B. dendrobatidis* isolate separately.

**Scenario 3:** Sequential *B. dendrobatidis* exposure: Finally, we examined the ability of the three probiotic types to inhibit two *B. dendrobatidis* isolates encountered in series by randomly selecting two of the three *B. dendrobatidis* isolates. We assumed that the two isolates are not encountered simultaneously as co-occurrence of two *B. dendrobatidis* isolates may modify their growth rates and/or a bacterial strain's ability to inhibit them. For each iteration, we selected a random multigenus and single-genus consortium, followed by a randomly selected single strain member from the multigenus consortium. Individual inhibition scores for these three groups were then extracted for both selected *B. dendrobatidis* isolates (i.e., probiotic ID was kept consistent over both pathogen isolates). We calculated the probability that the multigenus consortium would yield superior inhibition to the single-genus consortium and single bacterial strain across both *B. dendrobatidis* isolates, and the probability that all three probiotic types would yield >50% inhibition.

## 3 | RESULTS

### 3.1 | BdGPL inhibition within and among bacterial genera

We assayed the ability of 54 bacterial strains from 10 genera to modify the growth rates of 10 BdGPL isolates. Mean inhibition scores ranged from 100 (complete inhibition of growth) to −225 (strong facilitation of growth). There were no significant differences among genera in mean proportion of BdGPL isolates inhibited (binomial GLMM;  $\chi^2_9 = 8.12$ ,  $p = .52$ ; Figure 1; Table 2). Six strains from four genera showed at least weak inhibition across all 10 BdGPLs, whilst five strains from four genera facilitated the growth of all 10 *B. dendrobatidis* isolates (Table S1). We did not find a significant correlation between genetic distance and similarity of inhibition profiles (Mantel test  $r = -.027$ ,  $p = .77$ ).

We detected considerably more variation in inhibition scores among bacterial strains within genera than among genera (Figure 1). Variation among bacterial strains within genera explained 87.9% [95% credible interval (CRI) 80.25%–94.47%] of the variation in

**TABLE 2** Mean proportion of 10 *BdGPL* isolates for which at least weak inhibitory capability was observed, averaged over all bacterial strains in a genus

Genus	Number of Isolates	Mean Proportion <i>BdGPL</i> Inhibition	95% CI
<i>Acinetobacter</i>	6	0.33	0.1–0.64
<i>Chryseobacterium</i>	8	0.50	0.25–0.75
<i>Citrobacter</i>	3	0.67	0.24–0.95
<i>Comamonas</i>	4	0.70	0.32–0.94
<i>Enterobacter</i>	7	0.73	0.44–0.92
<i>Microbacterium</i>	4	0.40	0.1–0.76
<i>Sanguibacter</i>	3	0.63	0.22–0.94
<i>Serratia</i>	6	0.47	0.19–0.76
<i>Staphylococcus</i>	4	0.73	0.35–0.96
<i>Stenotrophomonas</i>	9	0.49	0.25–0.73

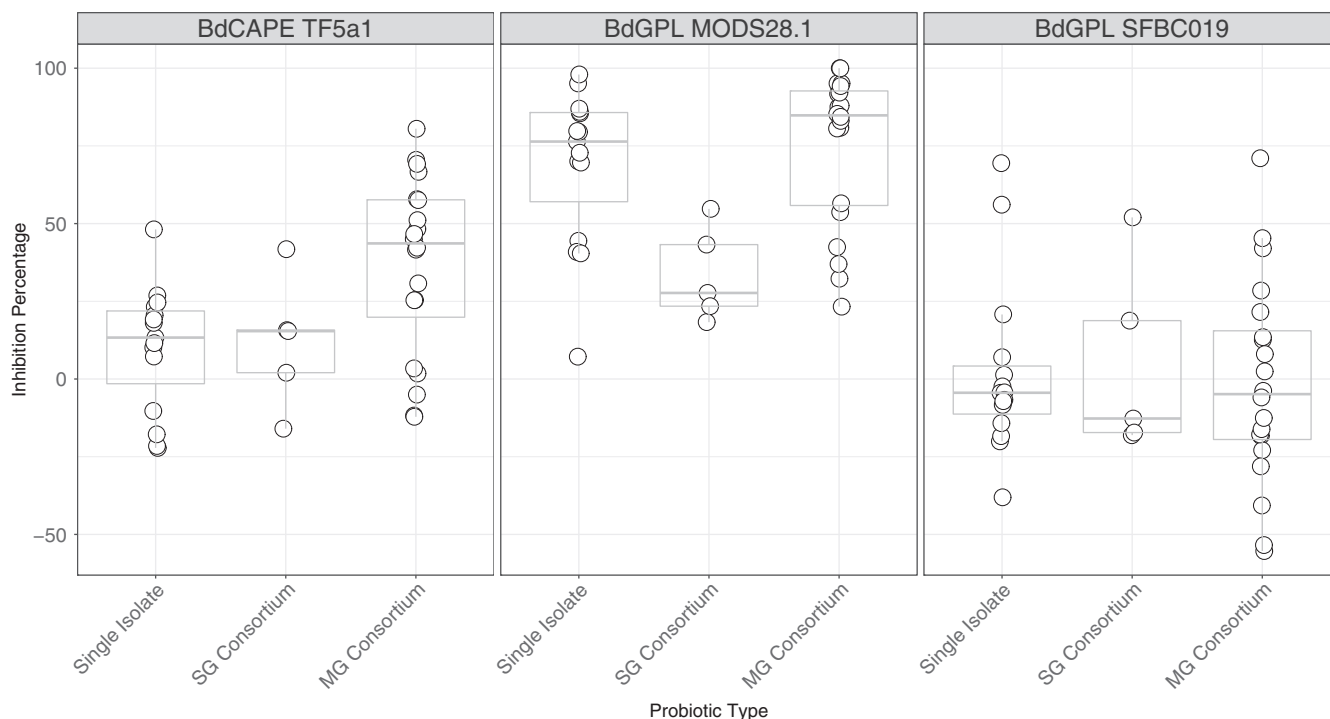
95% CI: 95% confidence intervals from an overdispersion-corrected binomial GLM.

*BdGPL* inhibition scores compared to just 0.6% [0.007%–3.8%] for bacterial genus. *BdGPL* isolate explained 3.9% [0.1%–8.7%] of the variation in inhibition scores. Heatmap hierarchical clustering of inhibition scores revealed two isolates that demonstrated predominantly enhanced growth in the presence of bacterial filtrates (JEL423 and AUL2; Figure 2). In one case, *B. dendrobatidis* isolates from similar locations (i.e., CORN isolates from Cornwall) exhibited similar

clustering of inhibitions scores, whereas in another case (i.e., AUL isolates from the Pyrenees) these showed markedly different inhibition fingerprints (Figure 2).

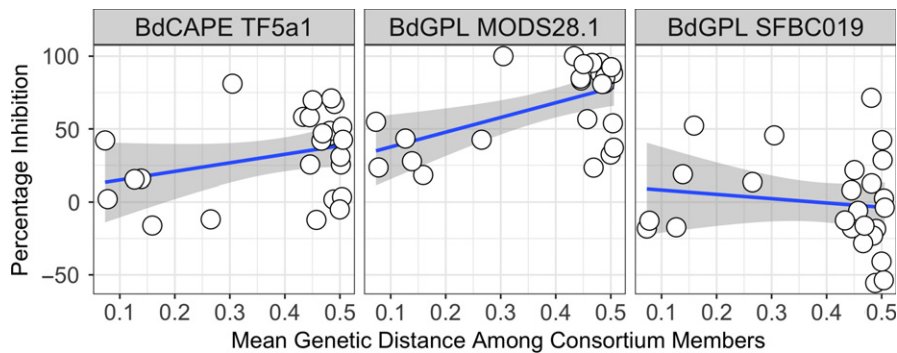
### 3.2 | Multistrain consortia as tools for pathogen mitigation

Consortia containing strains from multiple genera exhibited significantly higher mean inhibition scores compared to single-genus consortia when marginalizing with respect to *B. dendrobatidis* isolate (multigenus consortia mean inhibition: 36.88%; single-genus consortia mean: 16.9%; 95% CRI of difference 4.12%–36.52%,  $p_{MCMC} = 0.02$ ; Figure 3). Multigenus consortia had a 61% probability of demonstrating stronger inhibition than the mean of their single composite bacterial strains, which was significantly higher than the corresponding probability for single-genus consortia (26.6%, mean difference 39.4% [95% credible interval 11.2%–65.1%],  $p_{MCMC} = 0.01$ ). Mean genetic distance among members of multigenus consortia was significantly higher than among members of single-genus consortia (multigenus mean distance = 0.45, single-genus mean = 0.11,  $t = -15.5$ ,  $p < .001$ ). Consortia with higher mean genetic distance elicited significantly higher inhibition scores for *B. dendrobatidis* isolates *BdCAPE* TF5a1 and *BdGPL* MODS28.1 ( $p_{MCMC} = 0.009$ ), but not for *BdGPL* SFBC019, which had a significantly different slope to the other two *B. dendrobatidis* isolates (Figure 4,  $p_{MCMC} = 0.01$ ).



**FIGURE 3** Inhibition scores for single-genus (SG) and multigenus (MG) consortia across three *Batrachochytrium dendrobatidis* isolates (*BdGPL* MODS28.1, *BdGPL* SFBC019 and *BdCAPE* TF5a1). A positive value represents inhibition of *B. dendrobatidis* growth, and a negative value indicates enhanced growth of *B. dendrobatidis*. Points have been jittered for display purposes





**FIGURE 4** Relationship between mean genetic distance among consortium members and *Batrachochytrium dendrobatidis* inhibition score. We detected a significant positive relationship between genetic distance and inhibition percentage for BdCAPE TF5a1 and BdGPL MODS28.1 but not BdGPL SFBC019. Fitted lines and shaded areas are mean and 95% confidence intervals from a linear model fit

### 3.3 | Probiotic consortium randomizations

**Scenario 1:** Our randomization tests revealed that multigenus consortia gave higher inhibition than single-genus consortia in 69.4% of cases when averaging over all *B. dendrobatidis* isolates (null expectation 50%,  $p_{\text{RAND}} < 0.001$ ). Multigenus consortia were more likely to produce inhibition greater than 50% (strong inhibition) (38.1% of iterations) compared to single-genus consortia (13.9% of iterations,  $p < .001$ ) and outperformed a randomly chosen single bacterial strain in 61% of cases (null expectation 50%,  $p_{\text{RAND}} < 0.001$ ). Mean inhibition for all multigenus consortia across all *B. dendrobatidis* isolates was 36.7%, compared to 16.47% for single-genus consortia.

**Scenario 2:** When considering *B. dendrobatidis* isolates individually, multigenus consortia outperformed single-genus consortia and single bacterial strains for only two of the three isolates (BdGPL MODS28 and BdCAPE TF5a1, but not for BdGPL SFBC019; Figure 5a). This pattern was also evident when determining the probability of yielding >50% inhibition by consortia (Figure 5b).

**Scenario 3:** We also tested the ability of both multigenus and single-genus consortia to inhibit the growth of two different *B. dendrobatidis* isolates in series, as individuals in a single location may be exposed to multiple variants of a pathogen (Goka et al., 2009; Jenkinson et al., 2016; Rodríguez et al., 2014; Schloegel et al., 2012), or strong spatial structure of the pathogen and high host dispersal may expose individuals to multiple pathogen variants consecutively. Applying the same multigenus consortium to two different randomly chosen *B. dendrobatidis* isolates in series achieved stronger inhibition than single-genus consortia in 49.4% of cases (null expectation 25%,  $p_{\text{RAND}} < 0.001$ ). This compared to only 7.9% of cases where single-genus consortia exhibited superior inhibition for both *B. dendrobatidis* isolates. Multigenus consortia exhibited strong inhibition (>50%) for both isolates in 14.7% of cases, compared to zero cases where single-genus isolates did so. Applying a single bacterial strain instead of a single-genus or multigenus consortium resulted in strong inhibition for both *B. dendrobatidis* isolates in only 4% of cases (Figure 5c). The full results of these randomizations, including confidence intervals for tests, can be found in Table S2.

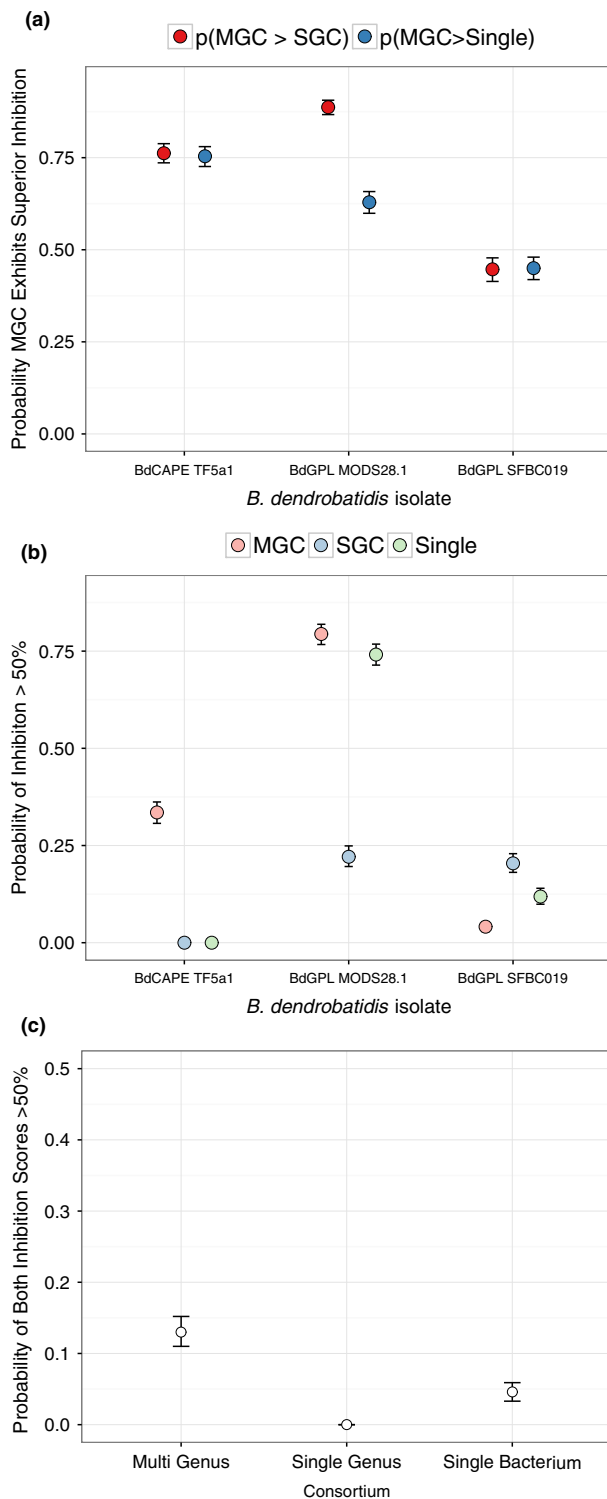
## 4 | DISCUSSION

The principal objectives of this study were twofold (i) to determine whether certain genera of bacteria are better able to inhibit a broad range of *BdGPL* isolates; and (ii) to examine the relative effectiveness of single bacteria and bacterial consortia to inhibit multiple isolates of *B. dendrobatidis*. We found no evidence of variation among bacterial genera in their ability to exhibit broad-range inhibition across multiple *BdGPL* isolates. There was considerable within-genus variation in inhibitory capabilities of bacteria compared to between-genus variation, meaning genus is not a reliable indicator of anti-*B. dendrobatidis* capabilities across multiple isolates of this pathogen. Furthermore, our data suggested consortia can provide superior *B. dendrobatidis* inhibition compared to individual bacteria and that this is contingent on consortium taxonomic diversity, but critically this pattern is not uniform across pathogen isolates. Our results have important implications for developing effective strategies for designing probiotic therapies to mitigate lethal infectious disease.

### 4.1 | BdGPL inhibition within and among bacterial genera

We found no evidence of systematic variation among bacterial genera in their ability to inhibit multiple *BdGPL* isolates. In our data, the principal source of variance in inhibition was among bacterial strains, with the number of strains demonstrating broad-spectrum facilitation of *BdGPL* being approximately equal to the number exhibiting broad-scale inhibition of the pathogen. These data support previous work suggesting *B. dendrobatidis* inhibition capability is distributed widely across bacterial genera (Antwis et al., 2015; Becker et al., 2015; Bletz, Myers, et al., 2017); several strains demonstrated at least weak inhibition for all 10 *BdGPL*s but were spread across multiple genera with no clear pattern. That there is clear functional redundancy among genera in this host-protective trait suggests it is not prudent to focus on any one genus in the search for beneficial probiotics (Becker et al., 2015), as highly divergent microbial communities can still possess similar functional traits (e.g., Bletz et al., 2016; Bletz, Perl, 2017).

We identified one *BdGPL* isolate that was significantly prone to inhibition (08MG04) and a further two isolates that demonstrated



**FIGURE 5** Randomization results examining the relative efficacy of different probiotic strategies. (a) the probability of multigenus consortia (MGC) yielding higher inhibition compared to single-genus consortia (SGC) or a single bacterial strain (Single); (b) the probability of MGC, SGC or single bacteria yielding inhibition > 50% when applied to each of three *Batrachochytrium dendrobatidis* isolates; (c) the probability of an individual consortium type yielding >50% inhibition when applied to two randomly chosen *B. dendrobatidis* isolates in series

strong resistance to inhibition (i.e., facilitated growth; AUL2 and JEL423). The phenomenon of *BdGPL* growth facilitation has been described previously for single pathogen isolates (Becker et al., 2015; Bell et al., 2013), but crucially, our results suggest that a bacterial strain's ability to facilitate the growth of *B. dendrobatidis* extends across a broad suite of pathogen isolates. Thus, facilitation of *B. dendrobatidis* growth is not simply a rare phenomenon arising from specific *BdGPL*/bacterial combinations, and different *BdGPL* isolates may differ systematically in their growth rates when exposed to bacterial filtrates (see also Muletz-Wolz et al. 2017). It is unclear why some bacterial strains facilitate *B. dendrobatidis* growth, but one likely explanation is that certain bacterial metabolites can act as growth substrates or facilitators for fungi (Garbaye, 1994; Hardoim et al., 2015). In addition, different bacterial metabolites may alter the abiotic environment (e.g., pH) to confer different growth rates (Romanowski et al., 2011) or hormesis may occur whereby the growth of *B. dendrobatidis* is facilitated at low or intermediate concentrations of particular bacterial products (Bell et al., 2013).

Further research is required to determine whether a *BdGPL* isolates' susceptibility to inhibition or facilitation correlates with virulence, and how genotypic traits associated with the pathogen map on to inhibition profiles and taxonomic traits of bacteria. It would also be valuable to further explore the effects of coculturing bacteria with *B. dendrobatidis* prior to inhibition challenges, which may influence anti-*B. dendrobatidis* capabilities (Becker et al., 2015). In particular, *B. dendrobatidis* isolates that elicit particularly strong metabolites from bacteria (i.e., *B. dendrobatidis* isolates that are readily inhibited) could be used to prime probiotic bacteria to make these more effective at inhibiting other more resistant *B. dendrobatidis* isolates, such as AUL2 and JEL423 in this study.

## 4.2 | Consortium-based approaches to combatting fungal pathogens

Our results revealed that the relationship between taxonomic diversity of a probiotic consortium and its ability to inhibit *B. dendrobatidis* growth was not consistent across *B. dendrobatidis* isolates. Multigenus consortia outperformed both single-genus consortia and single bacterial strains in *B. dendrobatidis* inhibition, and were far more likely to produce strong inhibition of 50% or greater, but this is true only for two of the three pathogen variants. Previous work has demonstrated a link between consortium species richness and *B. dendrobatidis* inhibition but only for a single pathogen isolate (Loudon et al., 2014; Piovia-Scott et al., 2017). Our data suggest that this pattern may not be general, with marked variation among pathogen isolates in their susceptibility to multigenus consortia.

That said, the general relationship (for two of the three pathogen variants) between inhibition and consortium diversity was in the expected direction; low community relatedness (i.e., high community dissimilarity) and high species richness both increase the resistance of a bacterial community to pathogenic "invaders" (Eisenhauer,

Scheu, & Jousset, 2012; Eisenhauer et al., 2013; Jousset, Schmid, Scheu, & Eisenhauer, 2011). That multigenus consortia can provide superior inhibition for some pathogen variants is suggestive of synergistic effects, whereby the combined pool of metabolites from multiple bacteria inhibits *B. dendrobatidis* more strongly than the individual strains (Loudon et al., 2014). Superior inhibition from consortia, rather than single strains, may arise as a by-product of the interference competition over resources created by coculture (Scheuring & Yu, 2012). Bacteria that are weak inhibitors when used individually (as in this study) could increase the overall inhibitory power of a consortium by creating a competitive environment that favours greater production of antifungal compounds.

We found that one of the three *B. dendrobatidis* isolates (BdGPL SFBC019) was not susceptible to inhibition from more diverse consortia as exhibited the other two pathogen variants (BdCAPE TF5a1 and BdGPL MODS 28.1). That *B. dendrobatidis* isolate can alter the strength of the relationship between consortium diversity and inhibition is a highly novel finding. BdGPL SFBC019 appears largely resistant to inhibition irrespective of whether individual bacteria or consortia are used, with individual bacterial inhibition scores that were often negative (Figure 3). This suggests resistance to inhibition from single strains may not necessarily be overcome by the putative synergistic effects from coculturing bacteria, in the same way that total microbial communities (along with other anti-*B. dendrobatidis* factors associated with the skin) of amphibians may not always be resistant to particular variants of the pathogen (Antwis & Weldon, 2017). The underlying cause for this variation is unclear as our data suggest this variation in consortia-based inhibition does not appear to correlate with *B. dendrobatidis* lineage. In addition, the results of the single strain challenges with 10 BdGPL isolates showed all four isolates from one locality in the UK (CORN isolates; Table 1; Figure 2) showed similar levels of inhibition across all bacterial strains, whereas the two isolates from the same locality in France (AUL isolates; Table 1; Figure 2) exhibited markedly different inhibition profiles. This suggests even pathogen isolates collected from the same host species and locality have the potential to exhibit markedly different responses to bacterial probiotics. More work is required to determine the relative inhibition profiles of multiple *B. dendrobatidis* isolates challenged with single- and multibacterial probiotics across a spectrum of diversity and to determine the mechanisms driving the responses of *B. dendrobatidis* variants to these.

In the study presented here, some metabolites (and other bacterial products) will have been carried over from bacterial strains whilst constructing single- and multispecies consortia, and it is also possible that after 12 hr of coculture, the proportions of bacteria in the multispecies consortia were not equal. Thus, it would be beneficial to determine how inhibition profiles of mixed-species consortia alter over time and whether this can be optimized for the mitigation of wildlife disease. Similarly, understanding the response of the host microbiome to inoculation by probiotics, and concurrent factors that determine the longevity of probiotics on the skin of amphibians, would provide significant steps forward in developing effective treatments.

## 4.3 | Conclusion

Our work has highlighted that different isolates of a lethal wildlife pathogen can vary in their susceptibility to probiotic bacteria, meaning we cannot expect probiotic effectiveness to be uniform across the genetic or phenotypic landscape of the pathogen. That said, higher diversity (both in terms of richness and phylogeny) of probiotic consortia may provide greater protective capabilities against pathogens than individual bacteria, although some *B. dendrobatidis* isolates may be largely resistant to the majority of bacterial probiotics, and using bacterial consortia may not overcome this. These patterns are informative with respect to potential strategies for the application of bacterial probiotics to mitigate *B. dendrobatidis* and other wildlife pathogens. Conservationists might not always know which particular *B. dendrobatidis* variant is infecting a local population, preventing targeted application of known strong inhibitors for that variant (Muletz-Wolz et al. 2017), and both time and expense may prevent the establishment of such a database de novo if a probiotic intervention is required rapidly. Therefore, we must employ strategies that maximize the chance of successful inhibition in the absence of perfect knowledge of the pathogen. Although multigenus consortia did not always outperform single-genus consortia or single bacteria strains, our data did reveal that these consortia have the highest probability of "strong" inhibition of >50% if applied "naively" without knowledge of the pathogen variant. This finding is important; human-mediated spread of *B. dendrobatidis* through the amphibian trade (Fisher & Garner, 2007) means we cannot assume that local populations will be exposed to only one pathogenic variant. Future work will expand this study to test multigenus consortia against a broader range of pathogen isolates to determine the generality of this pattern, in addition to identifying the inhibitory capabilities of consortia constructed from bacteria with medium to strong inhibition profiles. It would be particularly interesting to combine whole-genome sequencing of the pathogen with inhibition data from single bacterial strains and consortia to assess whether closely related pathogen isolates are more likely to show similar responses, or lack thereof, to bacterial consortia. Despite the potential merits of multigenus consortia for mitigating single and multiple *B. dendrobatidis* variants, it remains to be determined how readily these consortia will be able to colonize the host skin in vivo. This is crucial for quantifying how applicable inhibition measures derived in vitro are to real-world scenarios. Additionally, although we tend to treat bacterial inhibition scores as fixed traits, this ignores the ability of genetic recombination among *B. dendrobatidis* lineages to modify the relationship between bacterial metabolites and pathogen growth rates. Even the application of probiotics themselves may represent a strong selective pressure favouring genetic variants of *B. dendrobatidis* that lack susceptibility to those probiotics. Although several trials have demonstrated the potential for probiotic prophylaxis against *B. dendrobatidis*, we still lack the requisite data to measure selection caused by those trials on the pathogen. In vitro experimental evolution assays between pathogen and bacteria may prove the most powerful means for detecting such patterns.

## ACKNOWLEDGEMENTS

This study was funded by a North-West University Postdoctoral Research Fellowship and a University of Salford Research Pump Priming Fund awarded to REA. XAH was funded by an Institute of Zoology Research Fellowship. The authors would like to thank Prof Richard Preziosi and Prof Trenton Garner for additional provision of consumables, and Prof Ché Weldon, Prof Trenton Garner and Prof Matthew Fisher for access to *Batrachochytrium dendrobatidis* isolates used in this study.

## DATA ACCESSIBILITY

All code and data to reproduce the results in this study will be uploaded to FigShare upon publication at <https://doi.org/10.6084/m9.figshare.5633821>.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTION

The study was conceived by R.A. and X.H. The data were collected by R.A. The data were analysed by X.H. The manuscript was written by R.A. and X.H. Both authors contributed equally to this manuscript.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Antwis RE, Harrison XA. Probiotic consortia are not uniformly effective against different amphibian chytrid pathogen isolates. *Mol Ecol*. 2018;27:577–589. <https://doi.org/10.1111/mec.14456>